Prodesse® ProParaflu®+ Assay

Instructions for Use

For detection and discrimination of Parainfluenza 1 Virus, Parainfluenza 2 Virus and Parainfluenza 3 Virus.

IVD

Rx Only

Σ

100

-70 °C

CE

REF

303677

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Intended Use

The Prodesse® ProParaflu®+ assay is a multiplex Real-Time PCR (RT-PCR) *in vitro* diagnostic test for the qualitative detection and discrimination of Parainfluenza 1 Virus, Parainfluenza 2 Virus and Parainfluenza 3 Virus (HPIV-1, HPIV-2 and HPIV-3) nucleic acids isolated and purified from nasopharyngeal (NP) swab specimens obtained from individuals exhibiting signs and symptoms of respiratory tract infections. This assay targets the conserved regions of the Hemagglutinin-Neuraminidase (HN) gene of HPIV-1, HPIV-2 and HPIV-3, respectively. The detection and discrimination of HPIV-1, HPIV-2 and HPIV-3 nucleic acids from symptomatic patients aid in the diagnosis of human respiratory tract parainfluenza infections if used in conjunction with other clinical and laboratory findings. This test is not intended to detect Parainfluenza 4a or Parainfluenza 4b Viruses.

Negative test results are presumptive and should be confirmed by cell culture. Negative results do not preclude Parainfluenza 1, 2 or 3 virus infections and should not be used as the sole basis for treatment or other management decisions.

Summary and Explanation

Human Parainfluenza viruses are negative-sense single stranded RNA viruses surrounded by fusion protein and hemagglutinin–neuraminidase glycoprotein “spikes” on the surface[1]. There are four serotypes of HPIV (1 through 4). HPIVs are the second major causative agents of lower respiratory tract infections in infants and young children[2]. Symptoms of infection with HPIV include common cold with fever, croup, bronchiolitis, and pneumonia[3]. Re-infections with HPIVs are common throughout life, especially in elderly and immunocompromised patients. Each of the four serotypes has distinct clinical and epidemiological features. HPIV-1 and HPIV-2 are the leading causes of croup in children while HPIV-3 is more often associated with bronchiolitis and pneumonia. HPIV-4 has a low recovery rate in cell culture, reportedly causes mild respiratory disease and historically has not been included in the routine respiratory virus testing in most clinical virology laboratories[4]. The incubation period for HPIVs is usually 1 to 7 days[3]. Transmission of HPIVs occurs through spread of respiratory secretions from infected persons or contact with contaminated surfaces or objects. HPIVs can remain infectious in aerosols for at least an hour. Internationally, HPIV has a worldwide distribution and epidemics are known to occur, particularly with HPIV-1. Approximately 41,000 individuals per year are admitted to the hospital in the U.S. for parainfluenza infections[5].

Principles of the Procedure

The ProParaflu+ assay enables detection and differentiation of Parainfluenza 1 Virus, Parainfluenza 2 Virus, Parainfluenza 3 Virus and a Universal Internal Control.

An overview of the procedure is as follows:

1. Collect nasopharyngeal swab specimens from symptomatic patients using a polyester, rayon or nylon tipped swab and place into viral transport medium (refer to *Materials Required but not Provided*).
2. Add a Universal Internal Control (UIC) to every sample to monitor for inhibitors present in the specimens.
3. Perform isolation and purification of nucleic acids using a MagNA Pure LC System (Roche) and the MagNA Pure Total Nucleic Acid Isolation Kit (Roche) or a NucliSENS easyMAG System (bioMérieux) and the Automated Magnetic Extraction Reagents (bioMérieux).
4. Add purified nucleic acids to ProParaflu+ Supermix along with enzymes included in the ProParaflu+ assay Kit. The ProParaflu+ Supermix contains oligonucleotide primers and target-specific oligonucleotide probes. The primers are complementary to highly conserved regions of genetic sequences for these respiratory viruses. The probes are dual-labeled with a reporter dye attached to the 5'-end and a quencher dye attached to the 3'-end (see table below).
5. Perform reverse transcription of RNA into complementary DNA (cDNA) and subsequent amplification of DNA in a Cepheid SmartCycler II instrument. In this process, the probe anneals specifically to the template followed by primer extension and amplification. The ProParaflu+ assay is based on Taqman reagent chemistry, which utilizes the 5' – 3' exonuclease activity of the Taq polymerase to cleave the probe thus separating the reporter dye from the quencher. This generates an increase in fluorescent signal upon excitation from a light source. With each cycle, additional reporter dye molecules are cleaved from their respective probes, further increasing fluorescent signal. The amount of fluorescence at any given cycle is dependent on the amount of amplification product present at that time. Fluorescent intensity is monitored during each PCR cycle by the real-time instrument.

Analyte	Gene Targeted	Probe Fluorophore	Absorbance Peak	Emission Peak	Instrument Channel
Parainfluenza 1 Virus	Hemagglutinin neuraminidase	FAM	495 nm	520 nm	FAM
Parainfluenza 3 Virus	Hemagglutinin neuraminidase	CAL Fluor Orange 560	540 nm	561 nm	TET
Parainfluenza 2 Virus	Hemagglutinin neuraminidase	CAL Fluor Red 610	595 nm	615 nm	Texas Red
Universal Internal Control	NA	Quasar 670	647 nm	667 nm	Cy5

Materials Provided

ProParaflu+ Assay Kit (Cat. #303677)

Note: For information on any hazard and precautionary statements that may be associated with reagents, refer to the Safety Data Sheet Library at www.hologic.com/sds**.**

Reagents	Description	Quantity/ Vials	Cap Color	Cat. #	Reactions/ Vials
ProParaflu+ Supermix	➲ Taq DNA polymerase ➲ 4 oligonucleotide primer pairs ➲ 4 oligonucleotide probes ➲ Buffer containing dNTPs (dATP, dCTP, dGTP, dTTP) ➲ $MgCl_2$ and stabilizers	1030 µL	Brown	HSM81	50 (2 vials provided)
M-MLV Reverse Transcriptase II	➲ 11.4 U/µL	36 µL	White	GLS32	100
RNase Inhibitor II	➲ 40 U/µL	120 µL	Green	GLS33	100
Parainfluenza RNA Control (Positive Control)	➲ Non-infectious *in vitro* transcribed RNA of specific viral sequences	500 µL	Red	HCT81	25
Universal Internal Control (UIC)	➲ Non-infectious *in vitro* transcribed RNA ➲ Non-infectious DNA plasmid	30 µL	Lilac	403097	100

Materials Required but not Provided

Plasticware and consumables

- Polyester, rayon or nylon tipped nasopharyngeal swabs
- RNase/DNase-Free 1.5 mL polypropylene microcentrifuge tubes
- Sterile RNase/DNase-free filter or positive displacement micropipettor tips
- MagNA Pure LC System Disposables (Reagent Tubs, Reaction Tips, Tip Trays, Cartridges) or easyMAG Disposables (Sample Strips and Tips)
- Biohit Pipette Tips for use with easyMAG System
- Greiner Break Four uncoated plate for use with easyMAG System
- Cepheid PCR reaction tubes, 25 µL
- Parafilm M or MagNA Pure LC Cartridge Seals

Reagents

Note: For information on any hazard and precautionary statements that may be associated with reagents, refer to the Safety Data Sheet Library at www.hologic.com/sds**.**

- Roche MagNA Pure LC Total Nucleic Acid Isolation Kit *(Roche Cat. No.03038505001)* for 192 isolations or bioMérieux NucliSENS easyMAG reagents *(Buffer 1 Cat. No. 280130, Buffer 2 Cat. No. 280131, Buffer 3 Cat. No. 280132, Magnetic Silica Cat. No. 280133, and Lysis Buffer Cat. No. 280134)*
- Micro Test M4 Viral Transport Medium *(Remel, Inc. Cat. No. R12500),* Micro Test M5 Viral Transport Medium *(Remel, Inc. Cat. No. R12515),* Micro Test M6 Viral Transport Medium *(Remel, Inc. Cat. No. R12530),* Micro Test M4RT Viral Transport Medium *(Remel, Inc. Cat. No. R12505),* Copan Universal Transport Medium (Copan Diagnostics, Inc., *Cat. No.* 330C), or BD Universal Viral Transport vial, 3mL *(Becton, Dickinson and Co. Cat. No. 220220)*
- Molecular Grade Water *(RNase/DNase Free)*

Equipment

- – 70ºC Freezer
- Roche MagNA Pure LC System with Software version 3.0.11 or bioMérieux NucliSENS easyMAG System with Software version 1.0.1 or 2.0
- Biohit multi-channel pipettor for use with easyMAG
- bioMérieux NucliSENS easyMAG Instrument
- Cepheid SmartCycler II Real Time Instrument with Dx Software version 1.7b, 3.0a or 3.0b
- Micropipettes (range between 1-10 µL, 10-200 µL and 100-1000 µL)
- Mini-centrifuge with adapter for Cepheid Reaction Tubes
- Cepheid cooling block
- Ice/Ice Bucket or -20°C Cold Block
- Biosafety Cabinet

Warnings and Precautions

- For *in vitro* diagnostic use only.
- Performance characteristics of this assay have only been determined with nasopharyngeal swab specimens.
- Limit the use of this product to personnel trained in the techniques of Real-Time PCR.
- Handle all specimens as if infectious using safe laboratory procedures such as those outlined in CDC/NIH *Biosafety in Microbiological and Biomedical Laboratories* and in the CLSI Document M29 *Protection of Laboratory Workers from Occupationally Acquired Infections*. Thoroughly clean and disinfect all surfaces with 10% bleach. Autoclave any equipment or materials that have contacted clinical specimens before discarding.
- Use micropipettes with aerosol barrier or positive displacement tips for all procedures.
- Always pre-plan, organize and segregate workflow. Workflow in the laboratory should proceed in a unidirectional manner, beginning in the Pre-Amplification Area and moving to the Amplification/Detection Area.
    - Begin pre-amplification activities with reagent preparation and proceed to specimen preparation.
    - Always dedicate supplies and equipment to a specified area; no cross-movement allowed between areas.
    - Do not use equipment and supplies used for reagent preparation for specimen preparation activities or for pipeting or processing other sources of target nucleic acid.
    - Keep all amplification supplies and equipment in the Amplification/Detection Area at all times.
    - Always wear disposable gloves in each area and change them before entering a different area.
    - Do not open sample tubes following PCR.
- Take care to preserve the purity of kit reagents. Avoid contamination from Positive Controls and specimens by following good laboratory practices.
- Do not use kit after its expiration date.
- Do not mix reagents with different lot numbers or substitute reagents from other manufacturers.
- Safety Data Sheets (SDS) are available on manufacturer's website at www.hologic.com.

Reagent Storage, Handling and Stability

- Store all reagents (opened and unopened) at ≤ – 70°C.
- Always check the expiration date on the reagent tubes. For Intermediate stock of the Universal Internal Control, use the expiration date of the originating stock control vial. Do not expose controls to more than one (1) freeze-thaw cycle.
- ProParaflu+ assay kits are shipped frozen, should arrive frozen and should be stored frozen after receipt. If the kit contents are not frozen, contact Customer Service for assistance.
- An internal study demonstrated that performance of ProParaflu+ Supermix, M-MLV Reverse Transcriptase II, and RNase Inhibitor II are not affected for up to 5 freeze-thaw cycles.
- Visually examine reagents for adequate reagent volume before beginning any test procedures.
- Protect the ProParaflu+ Supermix from light.
- **Controls and aliquots of controls must be thawed and kept on ice during preparation and use.**

Recommendation

Aliquoting of kit components to maintain less than 5 freeze/thaw cycles is recommended for labs with smaller batch sizes.

Specimen Collection, Handling and Storage

Collecting the Specimen

To obtain nasopharyngeal swab samples:

1. Insert a flexible-shaft polyester, rayon or nylon tipped swab containing a dry tip into one nostril and into the nasopharyngeal area.
2. Press the swab gently against the nasopharyngeal wall to allow the swab to absorb secretions.
3. Rotate the swab two to three times and withdraw it.
4. Place the swab into a tube containing 3 mL of viral transport medium (Remel M4, M4RT, M5, M6; Copan UTM; or Becton Dickenson UVT).
5. Break off the shaft of the swab and cap the tube.

Note

Using a smaller volume of the viral transport medium may result in inhibition.

Transporting Specimens

Ensure that when transporting human respiratory specimens, all applicable regulations for the transport of etiologic agents are met. Transport human respiratory specimens refrigerated at 2-8°C.

Storing Specimens

Store specimens refrigerated (2-8°C) for up to 72 hours before processing. Store any leftover specimens at ≤ – 70°C. If retesting a frozen specimen, thaw specimen quickly (1 to 2 minutes) in a 37°C water bath and immediately place on ice or thaw specimen on ice.

Storing Purified Nucleic Acid

Store purified nucleic acids at ≤ – 70°C. They should be tested after no more than one (1) freeze-thaw cycle.

Note

Inadequate or inappropriate specimen collection, storage and transport are likely to yield false negative results.

Recommendation

Training in specimen collection is highly recommended because of the importance of specimen quality.

Reagent and Control Preparation

Reagents

Note

Prepare reagents from the Roche MagNA Pure LC Total Nucleic Acid Isolation Kit or the bioMérieux Automated Magnetic Extraction Reagents following the manufacturer's instructions.

Controls

Recommendation

- *For aliquots of the Positive Control and Intermediate Stock of the Universal Internal Control, use the expiration date of the originating stock control vial.*
- *Controls and aliquots of controls must be thawed and kept on ice/cold block at all times during preparation and use. It is recommended to prepare controls in a sample prep area, such as a Biological Safety Cabinet.*

Positive Control (PC)

Note

Include the Positive Control (the red cap vial) with each RT-PCR run.

1. Thaw Positive Control (the red cap vial) on ice.
2. Make 25 aliquots of 20 µL, label and store at ≤ –70°C. Ensure that aliquots do not undergo more than one (1) freeze-thaw cycle.
3. **The Positive Control is used at the provided concentration.**

Recommendation

Do not spike Positive Control with the Universal Internal Control. Do not take Positive Control through the nucleic acid isolation procedure.

Universal Internal Control (UIC)

1. Thaw Universal Internal Control (the lilac cap vial) on ice.
2. Create Intermediate stock tubes of the Universal Internal Control using the following dilution scheme:

26 µL Universal Internal Control	+	*65 µL RNase Inhibitor*	+	*2509 µL molecular grade water*	=	*2600 µL total volume*

3. Make aliquots of 110 µL, label, and store at ≤ –70°C (this is enough volume to add to 5 samples at 20 µL per sample). Make aliquots of larger or smaller volumes based on the number of samples expected to be processed in a single run. Ensure that aliquots do not undergo more than one (1) freeze-thaw cycle.
4. Add the appropriate volume of Intermediate stock of the Universal Internal Control to each sample prior to nucleic acid isolation (see ***Step 1*** of the ***Assay Procedure***).
5. **Save remaining RNase Inhibitor by re-freezing the leftover volume in the original tube for use in *Step 4* of the *Assay Procedure*.**

Negative Control (NC)

1. Use Viral Transport Medium as the Negative Control.
2. Add the appropriate volume of Intermediate stock of the Universal Internal Control to the Negative Control prior to nucleic acid isolation (see ***Step 1*** of the ***Assay Procedure***).

Extraction Control (EC)

Good laboratory practice recommends including a positive extraction control (e.g. previously characterized positive sample or negative sample spiked with a well characterized HPIV-1, HPIV-2 or HPIV-3 strain) in each nucleic acid isolation run. The extraction control should be treated as a sample during assay performance and analysis.

Assay Procedure

Assay Overview:

Get Ready: Create the Assay Protocol for the Cepheid SmartCycler instrument using the Dx Software *(first time only).*

1. ***Prepare the Samples and Negative Control.***
2. ***Isolate the Nucleic Acid –*** *MagNA Pure LC System using the Total Nucleic Acid Isolation (TNAI) Kit*

   OR
3. ***Isolate the Nucleic Acid –*** *NucliSENS easyMAG System using the Automated Magnetic Extraction Reagents.*
4. ***Set up the RT-PCR Reaction.***
5. ***Run the ProParaflu+ Assay.***
6. ***Print report.***

Note

- *Instructions provided for the Cepheid SmartCycler Real Time Instrument with Dx Software version 3.0a / 3.0b (Instructions for version 1.7b noted).*
- ***Do NOT deviate from the protocol settings defined in this section.***

Get Ready: Create the Assay Protocol for the Cepheid SmartCycler instrument using the Dx Software (first time only)

Note

- *The protocol is only created for first-time use; it does not need to be recreated with each sample run.*
- *Refer to SmartCycler Dx Software Operator Manual for assistance in defining assay protocols.*
- *To **Define** and **Edit** assay protocols, the user must have administrative access rights, otherwise the fields will be grayed out.*
- *Cepheid Dx Software interprets the data and reports the run as either VALID or INVALID, based on the results of the Negative Control. Enter the Positive Control and Extraction Control as if they were samples.*
- *Interpret the control results and determine if the run is VALID or INVALID. All Control criteria must be met in order for the run to be VALID (see **Interpretation of Results** section).*

1. Create the ProParaflu+ Assay protocol:

a. Launch the Cepheid Dx software application.

b. Click on the **Define Assay** box at the top of the screen.

c. Click on the **New Assay** box at the bottom of the screen.

d. Enter **ProParaflu+ Assay** for the assay protocol in the window that opens.

e. Click **OK**.

f. Enter **Thermocycler Parameters** in the Protocol section (bottom half of ***Define Assay*** screen).

Stage 1			**Stage 2**			**Stage 3** Repeat **5** times			**Stage 4** Repeat **45** times		
Hold			**Hold**			**2- Temperature Cycle**			**2- Temperature Cycle**		
Temp	Secs	Optics	Temp	Secs	Optics	Temp	Secs	Optics	Temp	Secs	Optics
42	**1800**	**OFF**	**95**	**600**	**OFF**	**95**	**30**	**OFF**	**95**	**10**	**OFF**
						55	**60**	**ON**	**55**	**60**	**ON**

Stages 5 – 10 remain UNUSED

2. Enter information in **BOLD** in the **Analysis Settings** tab as follows:
   a. Select **FTTC25** for the **Dye Set**.
   b. **Analysis Type: Qualitative** (default).
   c. **Customize Result Text: Target-based Result Text** (default).

Note *Gray boxes are default settings.*

Channel	Dye Name	Channel Name*	Usage	Curve Analysis	Thresh Setting	Manual Thresh	Auto Thresh	Auto Min. Cycle	Auto Max. Cycle	Valid Min. Cycle	Valid Max. Cycle	Bkgnd Sub	Bkgnd Min. Cycle	Bknd Max. Cycle	Boxcar Avg	EndPt Thresh	NC IC %	IC Delta
1	FAM*	**HPIV-1**	**Target****	**Primary Curve**	**Manual Thresh**	**60**	NA	5	10	13.0	**50**	On	5	**50**	0	**60**	NA†	NA
2	TET*	**HPIV-3**	**Target****	**Primary Curve**	**Manual Thresh**	**40**	NA	5	10	13.0	**50**	On	5	**50**	0	**40**	NA†	NA
3	TxR*	**HPIV-2**	**Target****	**Primary Curve**	**Manual Thresh**	**50**	NA	5	10	13.0	**50**	On	5	**50**	0	**50**	NA†	NA
4	Cy5*	**Universal Internal Control**	**Internal Control**	**Primary Curve**	**Manual Thresh**	**40**	NA	5	10	13.0	**50**	On	5	**50**	0	**40**	NA†	NA

**Dx 1.7b = Target*
*****Dx 1.7b = Assay***
† Dx 1.7b = 10

3. Enter information in **BOLD** in the **Control Settings** tab**.**
   a. Select **NC Fails if any target criterion is positive**.
   b. *Enter Positive Control and/or the Extraction Control as a sample. Do not use the Positive Control Settings. Enter **0** Replicates to inactivate Positive Controls PC1-3.* A 3079 error (Fluorescence Signal Too High) in the Positive Control invalidates the run, this is avoided if the Positive Control and/or the Extraction Control is entered as a sample and the results for each of the target channels are individually evaluated.
   c. *Use only one Negative Control (NC1). Enter **0** Replicates to inactivate the Negative Controls NC2 and NC3.*

Note
- *Gray boxes are default settings.*

Control ID	Control Name	Replicate	HPIV-1 Valid Min Cycle	HPIV-1 Valid Max Cycle	HPIV-1 EndPt Thresh	HPIV-3 Valid Min Cycle	HPIV-3 Valid Max Cycle	HPIV-3 EndPt Thresh	HPIV-2 Valid Min Cycle	HPIV-2 Valid Max Cycle	HPIV-2 EndPt Thresh	UIC +/-	UIC Valid Min Cycle	UIC Valid Max Cycle	UIC EndPt Thresh
PC1	Pos Cntrl 1	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10
PC2	Pos Cntrl 2	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10
PC3	Pos Cntrl 3	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10
NC1	**Neg Cntrl 1**	**1**	**13.0**	**50.0**	**60**	**13.0**	**50.0**	**40**	**13.0**	**50.0**	**50**	**+**	**15.0**	**50.0**	**40**
NC2	Neg Cntrl 2	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10
NC3	Neg Cntrl 3	0	13.0	45.0	10	13.0	45.0	10	13.0	45.0	10	+	13.0	45.0	10

4. Click the **Advanced** tab and select **Require Lot Number**. The **Probe Check Settings** tab, **Advance to New Stage** tab, and **Standards** tab are not used for the ProParaflu+ **Assay** protocol.
5. Select **Save Assay**.

1. Prepare the Samples, Extraction Control, and Negative Control (Pre-Amplification Area)

a. **Add Universal Internal Control to all samples.**

i. Thaw the appropriate number of aliquots of Intermediate stock of the Universal Internal Control (enough volume needed for each sample, the Extraction Control, and the Negative Control) on ice.

ii. Remove 180 µL of sample from the original sample tube and pipet into a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of sample directly into sample cartridge or sample vessel.

iii. Remove 180 µL of Extraction Control from the original sample tube and pipet into a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of Extraction Control directly into sample cartridge or sample vessel.

iv. Add 20 µL of Intermediate stock of the Universal Internal Control to each sample. **Pipet up and down a minimum of 5 times to mix** using a new pipet tip for each sample.

v. Keep tubes on ice.

vi. Store any remaining sample at ≤ -70°C.

b. **Add Universal Internal Control to the Negative Control.**

i. Include one (1) Negative Control in each run.

ii. Add 180 µL of Viral Transport Medium to a labeled 1.5 mL microcentrifuge tube. Alternately, pipet 180 µL of Viral Transport Medium directly into sample cartridge or sample vessel.

iii. Add 20 µL of Intermediate Stock of Universal Internal Control to the Viral Transport Medium. **Pipet up and down a minimum of 5 times to mix.**

iv. Keep tube on ice.

v. Discard remaining volume of Universal Internal Control – DO NOT reuse.

Do not reuse Universal Internal Control.

2. Isolate the Nucleic Acid (Pre-Amplification Area I) - MagNA Pure LC System using the Total Nucleic Acid Isolation (TNAI) Kit

a. **Start the instrument and software.**

i. Turn power on to the MagNA Pure LC instrument and then turn on the computer.

ii. Start the MagNA Pure LC software.

iii. From the *Main Menu* screen, select **Sample Ordering** and enter sample information in **Sample Name** column.

iv. Select the **Total NA Variable_elution_volume.blk** protocol.

v. Follow the software instructions and specify the number of samples.

vi. Type in **200 µL** for the sample volume and verify that 50 µL elution volume is selected.

vii. Select **Stage Setup** and the software automatically calculates the amount of each reagent that is required.

b. **Fill the reagent tubs**

Before starting the isolation procedure, fill all reagent tubs outside the instrument with the required volume of each reagent listed on the **Start Information** screen.

Note

Use only the reagent amount needed for the number of samples entered into the software. Reagents are not stable for long-term storage in tubs. Vortex Magnetic Glass Particles (MGPs) and load the exact amount of MGPs (as listed on the **Start Information** *Screen) into the instrument just before the run starts.*

c. **Load reagent tubs and disposables into the instrument**

Use the information on the **Start Information** screen to place all disposable plastics and reagent tubs necessary for the batch run on the Reagent/Sample Stage.

Recommendation

Use a colored "Positioning Frame" (provided with the TNAI kit) on the Reagent Tub Rack to help to correctly load reagents.

d. **Load the Samples, Extraction Control, and Negative Control into the MagNA Pure Sample Cartridge.**

i. Transfer 200 µL of each sample to individual wells in the MagNA Pure Sample Cartridge.

ii. Transfer 200 µL of the Extraction Control and Negative Control to different wells in the MagNA Pure Sample Cartridge.

iii. Cover cartridge with Parafilm or MagNA Pure LC Cartridge Seal and keep cartridge on ice until ready to load the instrument.

e. **Load the samples.**

Transfer cartridge containing the samples, Extraction Control, and Negative Control into the MagNA Pure LC instrument.

f. **Start the run**

i. Start the Batch Run by confirming the correct placement of all disposable plastics and reagents by mouse-clicking the respective text boxes on the **Start Information** screen.

ii. Select the **OK** button to start the automated isolation procedure. The instrument will automatically dispense all reagents and process the samples.

g. **Store the eluted total nucleic acid**

After completing the run, place the Storage Cartridge containing the eluted nucleic acids immediately on ice or transfer eluted nucleic acid to 1.5 mL tubes and store for longer durations at ≤ – 70°C.

Note

Do not store purified nucleic acids in the Storage Cartridge on the Cooling Unit 1.

3. Isolate the Nucleic Acid (Pre-Amplification Area I) - NucliSENS easyMAG System using the Automated Magnetic Extraction Reagents

a. **Start instrument and software.**

Turn power on to the easyMAG instrument and once the LED on the instrument turns green, turn on the computer and log into the software.

b. **Prepare the software for a run.**

To prepare for a run, touch "**Settings**"  in the main toolbar to default to the "Application Settings" icon and choose the following run settings:

Default Protocol: Generic 1.0.6 or 2.0.1 (for software version 1.0.1 or 2.0, respectively)
Run Name Prefix: N/A (leave as default)
Sample ID prefix: N/A (leave as default)
Sample Type: Primary (on-board lysis)
Default On-board Lysis Dispensing: Yes
Default On-board Lysis Incubation: Yes
Sample Addition Guidance: Off
Reagent Tracking: Off

c. **Input buffer information.**

Touch "**Instrument**"  to default to the "**Reagent Inventory**"  and input the buffer barcodes by first scanning the instrument position (A, B, C, or D) and then its corresponding buffer. For example, scan position A and then scan the bottle of Lysis buffer in that position and then move on to position B and its corresponding bottle.

d. **Create a worklist.**

i. Touch "**Daily Use**"  to default to the "**Define Extraction Request**"  icon and select the following settings:

Sample ID: Manually enter the sample name.
Matrix: Other
Protocol: Generic 1.0.6 or 2.0.1 (for software version 1.0.1 or 2.0, respectively)
Volume (mL): 0.20 (input volume of sample)
Eluate (µL): 55
Type: Primary
Priority: Normal

ii. Press **Enter** on the keyboard or touch "**New Extraction Request**"  after each manual sample addition. The settings above remain as the default settings for each subsequent entry as long as you do not navigate to other pages.

e. **Create a run and add samples from the worklist.**

Touch "**Organize Runs**"  and then "**Create Run**"  to display the *New Run Window*. In this screen, name the run appropriately and verify that the **auto-number** box is left unchecked (not selected) and that **Yes** is selected for both the On-Board Lysis Dispensing and On-Board Lysis Incubation options.

Touch **OK** and the *New Run Window* closes and the "**Organize Runs**" screen is displayed. Use the positioning arrows  to assign samples to the run. Touch "**Load Run**"  and select the run.

Touch "**Print worklist**"  to print the list. Use the worklist to keep track of the order of the samples to load into the sample vessels.

f. **Load samples, Extraction Control, Negative Control, and tips and barcode the samples strip(s).**

Add all of the 200 µL from each sample, Extraction Control, and Negative Control into the proper vessels of the sample well(s) as noted in the worklist. Insert tips into sample vessel(s) in the correct order as noted in the worklist and scan the sample strip(s) position on the instrument and then the sample strip itself. For example, scan position A and then the sample strip in that position, then B and then C, if necessary. After scanning the sample strip(s), the indicator changes from red to green on the screen.

g. **On-Board Lysis Dispensing.**

Once the samples and tips are loaded and the strips(s) scanned, close the lid and touch "**Dispense Lysis**"  . The instrument dispenses 2 mL of Lysis buffer and incubates for 10 minutes.

h. **Prepare the magnetic silica to add to the sample vessel.**

During the 10-minute lysis incubation, use the Biohit multi-channel pipettor to prepare the magnetic silica. Perform this procedure for each sample vessel used in the run (1, 2, or 3 times).

i. Set the pipettor to **Program 1** and place a Biohit pipette tip on position 1. Program 1 provides the means to aspirate and dispense 550 µL of liquid. The magnetic silica is prepared in a 1:1 ratio of Molecular Biology Grade Water to Magnetic Silica.

ii. Using Program 1 of the pipettor, press the **start** button to aspirate and then again to dispense 550 µL of water into a microcentrifuge tube. Vortex the tube of magnetic silica briefly to mix and use Program 1 of the pipettor to aspirate and then dispense 550 µL of magnetic silica into the same microcentrifuge tube as the water. Eject the tip, cap the tube, and vortex to mix.

iii. Set the pipettor to **Program 2** and place a Biohit pipette tip on position 1. Program 2 will transfer 8 volumes of the previous mix to the 8 vessels of a strip on an ELISA plate (1 strip/sample vessel). Press the **start** button to aspirate the mix. Press the **start** button again to dispense the remaining mixture back into the tube containing the mix to reset the pipette.

iv. Press the **start** button 8 separate times to dispense the remaining mix in each of 8 vessels of an ELISA plate strip and eject the tip.

v. After the 10 minute lysis incubation is done, set the pipettor to **Program 3** and place 8 Biohit pipette tips on the multi-channel pipettor (or however many samples are present in the specific sample strip). Make sure that the filter tips are very well connected with the multi-channel pipettor to prevent leakage errors. Program 3 first mixes the magnetic silica mixture in the ELISA plate and then aspirates it for delivery to the vessels of the sample strip where it will be mixed.

vi. Press the **start** button once and the pipette mixes the silica in the ELISA plate and then aspirates it for addition to the sample vessel. Verify that each tip has the same volume of silica mix before placing in the sample vessel. Place the pipettor over the sample vessel strip so the tips are below the liquid level of each sample and press the **start** button again to aspirate 800 µL out of each sample vessel and perform 3 mix cycles with 1000 µL. As it is mixing, be sure to hold the pipette steady below the liquid/air interface to avoid introducing bubbles to the sample.

vii. Repeat for each sample strip in the run.

i. **Start the run.**

Touch "**Start**"  to begin the run. The instrument performs 5 washes and heat and elute. The purified nucleic acids need to be transferred to appropriate storage tubes (1.5 mL microcentrifuge tubes) on ice within 30 minutes of extraction completion to avoid contamination by the magnetic silica stuck to the front wall of the sample vessel(s). Use immediately or store at -70°C.

4. Set up the RT-PCR Reaction (Pre-Amplification Area II)


Note

Start the SmartCycler ProParaflu+ assay run within 1 hour of making the RT-PCR Master mix.

a. **Thaw the Positive Control.**

i. Include the Positive Control with each run. Thaw one (1) aliquot of the Positive Control on ice.

ii. Keep tube on ice.

b. **Prepare the RT-PCR Master mix**


Note

The RT-PCR Master mix must be prepared FRESH for each RT-PCR run.

i. Calculate the amount of each reagent needed based on the number of reactions (samples + controls):

19.45 µL	ProParaflu+ Supermix
+0.30 µL	M-MLV Reverse Transcriptase II
+0.25 µL	RNase Inhibitor II
20.00 µL	per reaction

ii. Thaw the ProParaflu+ Supermix on ice and **mix by pipetting up and down a minimum of 5 times before use.**

iii. Remove M-MLV Reverse Transcriptase and RNase Inhibitor enzymes from the freezer and keep on ice during use. M-MLV Reverse Transcriptase should be spun down and pipetted from the top.

iv. Prepare the RT-PCR Master mix by combining the reagents listed above in a 1.5 mL microcentrifuge tube. **Pipet up and down a minimum of 5 times to mix.**

v. Keep the RT-PCR Master mix on ice and protected from light before adding to SmartCycler tubes.

c. **Add 20 µL of RT-PCR Master mix to the SmartCycler tubes.**

i. Load the required number of tubes into the Cepheid Cooling Block.

ii. Pipet 20 µL of the RT-PCR Master mix into the upper part of the SmartCycler tubes. Discard any unused RT-PCR Master mix.

d. **Add 5 µL of each sample's nucleic acid to individual SmartCycler tubes containing RT-PCR Master mix.**

i. After adding the sample's nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube. Use a new pipette tip for each sample.

e. **Add 5 µL of the Positive Control to a separate SmartCycler tube containing RT-PCR Master mix.**

i. After adding the Positive Control to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube using a new pipet tip for each control tube.**

ii. Close the tube.

iii. Discard remaining volume of Positive Control – DO NOT reuse.

Do not reuse Positive Control aliquot.

f. **Add 5 µL of the Extraction Control nucleic acid to a separate SmartCycler tube containing RT-PCR Master mix.**

i. After adding the Extraction Control nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube.

g. **Add 5 µL of the Negative Control nucleic acid to the last SmartCycler tube containing RT-PCR Master mix.**

i. After adding the Negative Control nucleic acid to the SmartCycler tube, **pipet up and down 2 to 3 times in the upper part of the tube.**

ii. Close the tube.

h. **Centrifuge all tubes**

i. Appropriately label the SmartCycler tubes on the caps.

ii. Centrifuge all tubes for 5 to 10 seconds using the Cepheid microcentrifuge specially adapted to fit the SmartCycler tubes.

iii. Return tubes to the cooling block.

i. **Keep the tubes on the Cooling Block before loading them into the SmartCycler instrument.**

5. Run the ProParaflu+ Assay (Amplification/Detection Area)

a. Create a new run by clicking **Create Run** at the top of the screen. The *Create Run* screen is displayed.

b. Under **Run Name** in the left panel of the *Create Run* screen, enter a unique run identifier.

c. Click the **Assay** arrow in the left panel of the *Create Run* screen and select the **ProParaflu+ Assay** protocol from the drop-down menu.

d. Under **Assay Information** in the left panel of the *Create Run* screen, enter the **Lot Number** and **Expiration Date** of the ProParaflu+ assay kit.

e. In the left panel of the *Create Run* screen, enter the number of specimens (including the Positive and Extraction Controls but excluding the Negative Control.) and click **Apply**. The **Site Table** is displayed and the SmartCycler Dx Software automatically selects the **I-Core** sites.

f. In the **Site Table** under the **Sample ID** column, enter the Sample Identifier or Positive Control Identifier for the appropriate I-Core sites.

g. Insert each reaction tube into an I-Core site of the SmartCycler by pressing down firmly on all tubes and close each lid. Verify that the Negative Control (NC1) is loaded into the correct I-Core site.

h. Select the **Start Run** button located at the bottom left corner of the screen. Verify that the LED is on for the appropriate I-Core sites.

6. Print Report

a. Click **Report** at bottom of screen to open the **Report Preview** screen.

b. Click **Print** at the top of the screen.

Interpretation of Control Results

Validation of Run

Note

You must interpret the Positive Control (PC) results and the Extraction Control (if included) results to determine whether the RT-PCR run and/or the extraction run are VALID; the SmartCycler Dx software automatically interprets the Negative Control result.

For a VALID Extraction run, the following conditions must be met:

Sample ID[1]	Assay Result	UIC Result	Warning / Error Code	Sample Type	UIC Ct	HPIV-1 or HPIV-2 or HPIV-3 Result	HPIV-1 or HPIV-2 or HPIV-3 Ct
Extraction Control	Positive	NA	**	SPEC	NA	POS	20-40
Neg Cntrl	Valid[2]	Pass		NC1	15-50	Valid	0

[1] Columns and data not used for interpretation are not included.

[2] (Typical) an Invalid assay will display Error Code 4098.

[**] Error Code 3079: Warning/Error Code 3079 is periodically observed with HPIV positives (Extraction Control, HPIV positive NP swab samples). Warning/Error Code 3079 occurs when the fluorescence (RFU) signal is too high. In this case, all results for the sample are reported by the Dx software as ND (Not Determined). When this code is observed for the Extraction Control, extraction run validity can be determined based on Ct values of the Extraction Control. The Extraction Control must have a Ct value between 20-40 in the HPIV-1 or HPIV-3 or HPIV-2 Ct column to be considered VALID.

For a VALID RT-PCR run, the following conditions must be met:

Sample ID[1]	Assay Result	UIC Result	Warning / Error Code	Sample Type	UIC Ct	HPIV-1 Result	HPIV-1 Ct	HPIV-3 Result	HPIV-3 Ct	HPIV-2 Result	HPIV-2 Ct
HPIV Cntrl[2]	Positive	NA	**	SPEC	NA	POS	20-40	POS	20-40	POS	20-40
Neg Cntrl	Valid[3]	Pass		NC1	15-50	Valid	0	Valid	0	Valid	0

[1] Columns and data not used for interpretation are not included.

[2] The Positive Control contains RNA for each of the Parainfluenza types (1 -3). The control needs to be valid/positive in all the channels, but the IC channel. Periodically bleedover may be observed in the IC (Cy5) channel, but this does not invalidate the run results.

[3] (Typical) an Invalid assay will display Error Code 4098.

[**] Error Code 3079: Warning/Error Code 3079 is periodically observed with HPIV positives (HPIV Positive Control). Warning/Error Code 3079 occurs when the fluorescence (RFU) signal is too high. In this case, all results for the sample are reported by the Dx software as ND (Not Determined). When this code is observed for the Positive Control run validity can be determined based on Ct values of the Positive Control. The Positive Control must have a Ct value between 20-40 in the HPIV-1, HPIV-3 and HPIV-2 Ct columns to be considered VALID.

Invalid Extraction Run

If the conditions for a valid extraction run are not met (i.e., the Extraction Control is not positive within the specified Ct range or the Negative Control is invalid), repeat the entire extraction run start from original sample(s) using a new Extraction Control and a new Negative Control (starting at ***Step 1*** of the ***Assay Procedure***).

Invalid RT-PCR Run

If the Positive Control is not positive within the specified Ct range but the Negative Control is valid, prepare all new reactions using remaining purified nucleic acids and a new Positive Control (starting with PCR at ***Step 4*** of the ***Assay Procedure***).

If the Negative Control is invalid, prepare all new extractions starting from original sample(s) using a new Extraction Control and a new Negative Control (starting at ***Step 1*** of the ***Assay Procedure***).

Interpretation of Specimen Results

The SmartCycler Dx software automatically determines the specimen results. The interpretation of the assay specimen results is as follows:

Sample ID[1]	Assay Result	UIC Result	Warning/ Error Code	HPIV-1 Result	HPIV-3 Result	HPIV-2 Result	Interpretation of Results
Sample ID	Negative	Pass		**NEG**	**NEG**	**NEG**	HPIV-1, -2 and -3 nucleic acid **not detected**
Sample ID	Positive	NA*		**POS**	NEG	NEG	HPIV-1 nucleic acid **detected**
Sample ID	Positive	NA*		NEG	**POS**	NEG	HPIV-3 nucleic acid **detected**
Sample ID	Positive	NA*		NEG	NEG	**POS**	HPIV-2 nucleic acid **detected**
Sample ID	Positive	NA*		**POS**	**POS**	NEG	HPIV-1 and HPIV-3 nucleic acid **detected** – multiple infections are rare, repeat testing from purified nucleic acid or re-test from original sample. **Other combinations (HPIV-1 and HPIV-2 or HPIV-2 and HPIV-3) may occur in the same manner.**
Sample ID	Unresolved	Fail		**NEG**	**NEG**	**NEG**	Unresolved – PCR inhibition or reagent failure. Repeat testing from the purified nucleic acid or re-test from original sample.
Sample ID	ND[2]	ND	3079[2]	ND	ND	ND	Not Determined – error code 3079
Sample ID	Invalid		4098[3]	ND	ND	ND	Not Determined – error code 4098

[1] *Columns and data not used for interpretation are not included*

[2] *Error Code 3079: Warning/Error Code 3079 is periodically observed. Warning/Error Code 3079 occurs when the fluorescence (RFU) signal is too high. In this case, all results for the sample are reported by the Dx software as ND (Not Determined). If a Ct value ≥13 is reported in any analyte column, results can be recorded as POSITIVE for that analyte.*

[3] *An Invalid assay run will display Error code 4098*

* *Detection of the Universal Internal Control in the Cy5 detection channel is not required for positive result. High viral load can lead to reduced or absent Universal Internal Control signal.*

Dual or Multiple Parainfluenza Infections

Dual or multiple parainfluenza infections are rare, and they may be artifacts of the SmartCycler Dx software due to signal bleed-over. It is required that repeat testing for these samples be performed starting from the purified nucleic acid, original sample, or a newly collected sample.

Not Determined Samples

If an assay result of **ND** (Not Determined) is reported with an instrument failure other than Warning/Error Code 3079, repeat testing from the purified nucleic acids (starting with PCR, see ***Step 4 (a)*** of the ***Assay Procedure***). Refer to the Cepheid Dx Software Operator Manual for interpretation of Warning Codes.

Quality Control

- Quality control requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard quality control procedures. It is recommended that the user refer to CLSI document C24-A3, *Statistical Quality Control for Quantitative Measurements: Principles and Definitions:* [Approved Guideline – Third Edition] or other published guidelines for general quality control recommendations. For further guidance on appropriate quality control practices, refer to 42 CFR 493.1205.
- Quality control procedures are intended to monitor reagent and assay performance.

Control Type	Used to Monitor
Positive	Substantial reagent failure including primer and probe integrity
Negative	Reagent and/or environmental contamination
Extraction	Failure in lysis and extraction procedure
Internal	PCR inhibition in individual samples and Reagent failure or process error

- Dilute the Universal Internal Control and test both the Positive Control and Universal Internal Control prior to running samples with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice recommends including a positive Extraction Control (not provided) in each nucleic acid isolation run. The Extraction Control should be treated as a sample.
- Never run the Positive Control through nucleic acid isolation.
- Always include a Negative Control *(containing Universal Internal Control)* and the Positive Control in each amplification/detection run performed.
- Failure of Controls (Positive, Negative and/or Extraction) invalidates the run and results should not be reported.
- If the Positive Control is not positive within the specified Ct range but the Negative Control is valid, repeat testing should be done starting from the purified nucleic acid and using a new aliquot of the Positive Control. If repeat results are still invalid, results should not be reported and testing should be repeated from the original sample or a new sample should be collected and tested.
- If the Extraction Control is not positive within the specified Ct range or the Negative Control is invalid, repeat testing should be done starting from the original sample and using a new Extraction Control and a new Negative Control. If repeat results are still invalid, results should not be reported and a new sample should be collected and tested.

Limitations

- **Once the RT-PCR Master mix is made, the run must be started within one hour.**
- This test does not detect Parainfluenza 4a or Parainfluenza 4b.
- Negative test results are presumptive and should be confirmed by cell culture. Negative results do not preclude Parainfluenza 1, 2 or 3 virus infections and should not be used as the sole basis for treatment or other management decisions.
- Dual or multiple parainfluenza infections are rare, and they may be artifacts of the SmartCycler Dx software due to signal bleed-over. It is required that repeat testing for these samples be performed starting from the purified nucleic acid, original sample, or a newly collected sample.
- Optimal assay performance requires strict adherence to the assay procedure describe in this insert.
- A trained health care professional should interpret assay results in conjunction with the patient's medical history, clinical signs and symptoms, and the results of other diagnostic tests.
- Analyte targets (viral nucleic acid) may persist *in vivo*, independent of virus viability. Detection of analyte target(s) does not imply that the corresponding virus(es) are infectious, or are the causative agents for clinical symptoms.
- The detection of viral nucleic acid is dependent upon proper specimen collection, handling, transportation, storage, and preparation (including extraction). Failure to observe proper procedures in any one of these steps can lead to incorrect results.
- There is a risk of false negative values resulting from improperly collected, transported, or handled specimens.
- There is a risk of false negative values due to the presence of sequence variants in the viral targets of the assay, procedural errors, amplification inhibitors in specimens, or inadequate numbers of organisms for amplification.
- A specimen yielding a negative result may contain respiratory viruses other than Parainfluenza 1, Parainfluenza 2 or Parainfluenza 3. A negative result should not be used as the sole basis for diagnosis, treatment or other management decisions.
- There is a risk of false positive values resulting from cross-contamination by target organisms, their nucleic acids or amplified product, or from non-specific signals in the assay.
- False negative results may occur due to loss of nucleic acid. The Universal Internal Control has been added to the test to aid in the identification of specimens that contain inhibitors to PCR amplification. The Universal Internal Control does not indicate whether or not nucleic acid has been lost due to inadequate collection, transport or storage of specimens.
- The ProParaflu+ assay may not generate reproducibly positive results when testing samples that have analyte concentrations lower than the LoD concentration, but higher than the assay cutoff concentration.
- The performance of the ProParaflu+ assay has not been established in immunocompromised patients.
- Positive and negative predictive values are highly dependent on prevalence. The assay performance was established during May 2008 to September 2009. The performance may vary depending on the prevalence and population tested.
- Some evidence of interference of HPIV detection at the LoD concentration was observed with throat lozenges, Relenza and Rebitol in the Interference Study, albeit these substances were tested at concentrations much higher than would be encountered *in vivo*.
- The ProParaflu+ assay has limited reactivity with the 2014 CAP sample ID2-08 and the 2015 CAP sample ID2-02. Sequencing analysis of the CAP samples revealed that the HPIV3 target sequences of the CAP samples match the sequence of HPIV3/Homo sapiens/PER/FLU8889/2007 strain in GenBank (GenBank Accession # KJ672604), and the limited reactivity is most likely due to a viral mutation in the probe binding region. Negative results may be obtained for samples containing this variant especially at low titers. If the ProParaflu+ assay does not indicate a positive result when an HPIV-3 infection is suspected, the specimen should be retested for HPIV-3 using an independent method (e.g. cell culture or molecular IVD).

Expected Values

In the US and around the world, infections with HPIV-1 and HPIV-2 occur in biennial alternating outbreaks during fall/early-winter where as HPIV-3 occurs throughout the year but generally peaks in spring[6,7]. Variables that affect the rate of positivity observed in respiratory testing include: the efficiency and timing of specimen collection, handling and transport of the specimen, the time of year, age of the patient, and local disease prevalence.

In the prospective ProParaflu+ assay clinical study, a total of 857 eligible nasopharyngeal (NP) swab specimens were tested from four U.S. clinical laboratories across the United States from May 2008 to September 2009. The number and percentage of HPIV-1, HPIV-2 and HPIV-3 RNA positive cases as determined by the ProParaflu+ assay, calculated by age group, are presented in the following table:

Age Group	Total N	Number HPIV-1 Positive By the ProParaflu+ Assay	Number HPIV-2 Positive By the ProParaflu+ Assay	Number HPIV-3 Positive By the ProParaflu+ Assay	Observed Prevalence HPIV-1	Observed Prevalence HPIV-2	Observed Prevalence HPIV-3
< 1 year	331	7	14	29	2.1%	4.2%	8.8%
1-5 years	249	9	9	11	3.6%	3.6%	4.4%
6-10 years	58	1	1	1	1.7%	1.7%	1.7%
11-15 years	61	0	1	0	0%	1.6%	0%
16-21 years	49	0	2	0	0%	4.1%	0%
> 21 years	109	0	1	3	0%	0.9%	2.8%
Total	857	17	28	43	2.0%	3.3%	5.0%

Performance Characteristics

Clinical Performance

The clinical performance of the ProParaflu+ assay was established during a prospective study at 4 U.S. clinical laboratories during May 2008 – September 2009. Specimens used in the study represented excess nasopharyngeal (NP) swab specimens that were prospectively collected from symptomatic individuals suspected of respiratory infection, and were submitted for routine analysis. Demographic details for this patient population are summarized in the following table.

Gender and Age Demographic Detail for ProParaflu+ Assay Prospective Study

Sex	Number of Subjects
Female	407 (47.5%)
Male	450 (52.5%)
Age (yrs)	
≤ 5 years	580 (67.7%)
6 - 21 years	168 (19.6%)
22 – 59 years	67 (7.8%)
≥ 60 years	42 (4.9%)

Performance of the ProParaflu+ assay was compared to the reference method of cell culture (rapid or traditional) followed by direct fluorescent antibody (DFA) screening and HPIV type identification.

A total of 857 eligible NP swab samples were tested with the ProParaflu+ assay and by culture across four clinical sites. Of the ProParaflu+ assay run on all eligible specimens, 99.2% (852/857) of these specimens were successful on the first attempt. The remaining 5 gave “Unresolved” results on the first attempt. Unresolved results occur when the sample is negative for all three HPIVs and the Internal Control, indicating potentially PCR-inhibiting samples. Of the 5 “Unresolved” specimens on the first attempt, 60.0% (3/5) gave a valid result on the second attempt. The remaining 2 were “Unresolved” on the second attempt and are not included in the analysis below. Both samples were culture negative.

Discrepant analysis for samples where ProParaflu+ assay and culture results were in disagreement was performed using RT-PCR with virus specific primers obtained from literature[8, 9] (and different from those used in ProParaflu+ assay) followed by bi-directional sequencing.

Prospective Study

Parainfluenza 1 Comparison Results

		Culture/DFA			
		Positive	**Negative**	**Total**	
ProParaflu+ Assay	**Positive**	16	1[a]	**17**	Sensitivity 88.9% (67.2% - 96.9%) 95% CI
	Negative	2[b]	838	**840**	Specificity 99.9% (99.3% - 100.0%) 95% CI
	Total	**18**	**839**	**857**	

[a]One (1) sample positive for HPIV-1 by bi-directional sequence analysis.
[b]Two (2) samples negative for HPIV-1 by bi-directional sequence analysis. One sample positive for HPIV-3 by ProParaflu+ assay and bi-directional sequence analysis.

Parainfluenza 2 Comparison Results

		Culture/DFA			
		Positive	Negative	Total	
ProParaflu+ Assay	Positive	26	2[a]	**28**	Sensitivity 96.3% (81.7% - 99.3%) 95% CI
	Negative	1[b]	828	**829**	Specificity 99.8% (99.1% - 99.9%) 95% CI
	Total	**27**	**830**	**857**	

[a]Two (2) samples positive for HPIV-2 by bi-directional sequence analysis.
[b]One (1) sample negative for HPIV-2 by bi-directional sequence analysis.

Parainfluenza 3 Comparison Results

		Culture/DFA			
		Positive	Negative	Total	
ProParaflu+ Assay	Positive	36	8[a]	**44**	Sensitivity 97.3% (86.2% - 99.5%) 95% CI
	Negative	1[b]	812	**813**	Specificity 99.2% (98.1% - 99.5%) 95% CI
	Total	**37**	**820**	**857**	

[a]Seven (7) samples positive for HPIV-3 and one (1) sample negative for HPIV-3 by bi-directional sequence analysis.
[b]One (1) sample negative for HPIV-3 by bi-directional sequence analysis.

Retrospective Study

Due to a minimal number of HPIV-1 positive samples, a retrospective study was also conducted using a total of 91 frozen NP swab samples that had been previously tested by direct DFA. Demographic details for this patient population are summarized in the following table.

Gender and Age Demographic Detail for ProParaflu+ Assay Retrospective Study

Sex	Number of Subjects
Female	40 (44.4%)
Male	50 (55.6%)
Age (yrs)	
≤ 5 years	81 (90.0%)
6 - 21 years	5 (5.6%)
22 – 59 years	2 (2.2%)
≥ 60 years	2 (2.2%)

Parainfluenza 1 Comparison Results

		DFA			
		Positive	Negative	Total	
ProParaflu+ Assay	Positive	24	0	**24**	Sensitivity 82.8% (65.4% - 92.4%) 95% CI
	Negative	5[a]	62	**67**	Specificity 100% (94.2% - 100%) 95% CI
	Total	**29**	**62**	**91**	

[a]Five (5) samples negative for HPIV-1 by bi-directional sequence analysis.

Reproducibility

The reproducibility of the ProParaflu+ assay was evaluated at 3 laboratory sites. Reproducibility was assessed using a panel of 9 simulated samples that included medium positive, low positive (near the assay limit of detection, ≥ 95% positive), and high negative (below the assay limit of detection, < 5% positive) samples. Panels and controls were tested at each site by 2 operators for 5 days (9 samples and 3 controls/run X 1 run/day/operator X 2 operators X 5 days X 3 sites = 360). Nucleic acid extraction on the test panel samples were carried out using either the Roche MagNA Pure LC System (Site #3) or the bioMérieux NucliSENS easyMAG System (Site #1 and Site #2). The overall percent agreement with the expected result for the ProParaflu+ assay was 97.8%.

	Panel Member ID	HPIV-1 high negative[a]	HPIV-1 low positive	HPIV-1 medium positive	HPIV-2 high negative[a]	HPIV-2 low positive	HPIV-2 medium positive	HPIV-3 high negative[a]	HPIV-3 low positive	HPIV-3 medium positive	Para Extraction Control	Para RNA Control			Negative Control[a]	Total % Agreement
												HPIV-1	HPIV-2	HPIV-3		
	Concentration	0.001 X LoD	2 X LoD	10X LoD	0.001 X LoD	2 X LoD	10X LoD	0.01 X LoD	2 X LoD	10X LoD	N/A	N/A			N/A	
Site 1	Agreement with Expected Result	10/10 100%	8/10 80%	9/9 100%	10/10 100%	9/9 100%	9/10 90%	10/10 100%	9/10 90%	10/10 100%	10/10 100%	10/10 100%			10/10 100%	**114/118 96.6%**
	Mean Ct Value	27.73	28.31	26.33	27.90	28.62	26.27	27.76	31.21	29.47	27.33	27.37	29.37	28.61	27.69	
	% CV	2.87	1.55	1.60	2.84	0.85	1.25	2.43	3.21	1.91	1.60	1.05	0.43	0.81	1.67	
Site 2	Agreement with Expected Result	8/10 80%	8/10 80%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%			10/10 100%	**116/120 96.7%**
	Mean Ct Value	28.59	28.47	26.12	28.83	28.91	26.61	28.30	31.64	29.51	27.56	23.86	26.09	25.23	28.68	
	% CV	1.36	1.72	1.26	2.80	1.31	1.83	1.18	2.17	2.66	2.47	1.48	1.12	0.92	1.15	
Site 3	Agreement with Expected Result	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%	10/10 100%			10/10 100%	**120/120 100%**
	Mean Ct Value	26.35	29.91	27.67	26.28	29.51	27.44	26.67	33.13	30.43	28.61	28.73	30.98	29.84	26.46	
	% CV	0.88	0.81	1.25	1.35	0.57	2.25	4.13	2.36	1.02	1.54	3.31	3.39	3.19	1.04	
	Total Agreement with Expected Result	28/30 93.3%	26/30 86.7%	29/29 100%	30/30 100%	29/29 100%	29/30 96.7%	30/30 100%	29/30 96.7%	30/30 100%	30/30 100%	30/30 100%			30/30 100%	**350/358 97.8%**
	95% CI	78.7% - 98.2%	70.3% - 94.7%	88.3% - 100%	88.6% - 100%	88.3% - 100%	83.3%- 99.4%	88.6% - 100%	83.3%- 99.4%	88.6%- 100%	88.6% - 100%	88.6% - 100%			88.6%- 100%	**95.6% - 98.9%**
	Overall Mean Ct Value	27.56	28.90	26.72	27.67	29.03	26.79	27.58	32.02	29.80	27.83	26.65	28.81	27.89	27.62	
	Overall % CV	3.88	2.87	2.95	4.55	1.57	2.54	3.68	3.61	2.42	2.75	8.13	7.49	7.39	3.54	

[a]Average Ct value for the Internal Control (IC)

An additional reproducibility study was performed to assess samples that were at an intermediate concentration, below the assay's LoD but above the "high negatives" tested during the original reproducibility study. Panels and controls were tested at each site by 2 operators for 5 days (3 samples and 3 controls/run X 1 run/day/operator X 2 operators X 5 days X 3 sites = 180). Nucleic acid extraction on the test panel samples were carried out using either the Roche MagNA Pure LC System (Site #3) or the bioMérieux NucliSENS easyMAG System (Site #1 and Site #2). The percent positive for the intermediate member across all sites was 56.7% for HPIV-1 (mean Ct = 35.1), 86.7% for HPIV-2 (mean Ct = 33.0), and 30.0% for HPIV-3 (mean Ct = 37.1). This result was expected as the intermediate concentration should be positive in the range of 5 - 95% as the samples were lower concentration than the LoD concentration (≥ 95% positive) and higher than the "high negative" concentration (< 5% positive).

	Panel Member ID	HPIV-1 intermediate	HPIV-2 intermediate	HPIV-3 intermediate	Para Extraction Control	Parainfluenza RNA Control			Negative Control[a]
						HPIV-1	HPIV-2	HPIV-3	
	Concentration	0.1 X LoD	0.1 X LoD	0.1 X LoD	N/A	N/A			N/A
Site 1	Agreement with Positive Result	4/10 40%	8/10 80%	1/10 10%	10/10 100%	10/10 100%			10/10* 100%
	Average Ct Value	35.5	33.3	36.9	27.9	28.8	30.4	29.5	28.4
	% CV	6.19	2.78	N/A	3.80	1.13	0.88	0.89	3.47
Site 2	Agreement with Positive Result	8/10 80%	10/10 100%	7/10 70%	10/10 100%	10/10 100%			10/10 100%
	Average Ct Value	34.4	32.2	37.3	27.5	29.0	30.6	29.9	27.7
	% CV	1.38	2.22	1.50	2.92	1.04	0.89	0.72	2.80
Site 3	Agreement with Positive Result	5/10 50%	8/10 80%	1/10 10%	10/10 100%	10/10 100%			10/10 100%
	Average Ct Value	35.9	33.7	35.8	28.6	29.7	31.5	30.3	27.9
	% CV	2.44	2.90	N/A	2.75	1.92	1.69	1.46	1.94
	Total Agreement with Positive Result	17/30 56.7%	26/30 86.7%	9/30 30.0%	30/30 100%	30/30 100%			30/30 100%
	95% CI	39.2% - 72.6%	70.3% - 94.7%	16.7% - 47.9%	88.7 – 100%	88.7 – 100%			88.7 – 100%
	Overall Average Ct Value	35.1	33.0	37.1	28.0	29.1	30.8	29.9	28.0
	Overall % CV	3.68	3.25	1.89	3.46	1.91	1.92	1.53	2.92

[a]Average Ct value for the Internal Control (IC)
*Agreement with Negative result

Analytical Sensitivity

The analytical sensitivity (limit of detection or LoD) of the ProParaflu+ assay was determined using quantified ($TCID_{50}$/mL) cultures of HPIV-1, HPIV-2 and HPIV-3 serially diluted in nasopharyngeal clinical matrix. Each viral strain was extracted using the Roche MagNA Pure LC instrument and tested in replicates of 20 per concentration of virus.

Analytical sensitivity (LoD), defined as the lowest concentration at which ≥ 95% of all replicates tested positive, ranged from $5 \times 10^1 – 10^2$ $TCID_{50}$/mL.

Virus	Strain	LoD Concentration
HPIV-1	C35	10^2 $TCID_{50}$/mL
HPIV-2	Greer	10^2 $TCID_{50}$/mL
HPIV-3	C 243	5×10^1 $TCID_{50}$/mL

Analytical Specificity

The analytical specificity of the ProParaflu+ assay was evaluated by testing a panel of 27 viruses, 24 bacteria, and 1 yeast strain representing common respiratory pathogens or flora commonly present in nasopharynx. Bacteria and yeast were tested at concentrations of 10^6 to10^8 CFU/mL. Viruses were tested at concentrations of 10^3 to10^6 $TCID_{50}$/mL. HPIV-1, 2 and 3 viruses were tested near and above LoD. Samples were extracted using the Roche MagNA Pure LC instrument and tested in triplicate. Analytical specificity of the ProParaflu+ assay was 100%.

Strains	Concentration	HPIV-1 (FAM)	HPIV-3 (TET)	HPIV-2 (Tex Red)
Parainfluenza Type 1	10^4 $TCID_{50}$/mL	+	-	-
Parainfluenza Type 1	$5x10^2$ $TCID_{50}$/mL	+	-	-
Parainfluenza Type 2	10^4 $TCID_{50}$/mL	-	-	+
Parainfluenza Type 2	$5x10^2$ $TCID_{50}$/mL	-	-	+
Parainfluenza Type 3	10^5 $TCID_{50}$/mL	-	+	-
Parainfluenza Type 3	$5x10^1$ $TCID_{50}$/mL	-	+	-
Parainfluenza Type 4	10^4 $TCID_{50}$/mL	-	-	-
Adenovirus 1/Adenoid 71	10^6 $TCID_{50}$/mL	-	-	-
Coronavirus 229E	10^6 $TCID_{50}$/mL	-	-	-
Coxsackie B4	10^4 $TCID_{50}$/mL	-	-	-
Coxsackie B5/10/2006	10^5 $TCID_{50}$/mL	-	-	-
Cytomegalovirus	10^4 $TCID_{50}$/mL	-	-	-
Echovirus 2	10^6 $TCID_{50}$/mL	-	-	-
Echovirus 3	10^5 $TCID_{50}$/mL	-	-	-
Echovirus 6	10^5 $TCID_{50}$/mL	-	-	-
Echovirus 11	10^6 $TCID_{50}$/mL	-	-	-
Enterovirus 68	10^3 $TCID_{50}$/mL	-	-	-
Enterovirus 70	10^3 $TCID_{50}$/mL	-	-	-
hMPV A2	10^4 $TCID_{50}$/mL	-	-	-
HSV Type 1 MacIntyre Strain	10^5 $TCID_{50}$/mL	-	-	-
HSV Type 2 G strain	10^5 $TCID_{50}$/mL	-	-	-
Human Rhinovirus 39	10^3 $TCID_{50}$/mL	-	-	-
Human Rhinovirus	10^4 $TCID_{50}$/mL	-	-	-
Influenza A/Port Chalmers	10^4 $TCID_{50}$/mL	-	-	-
Influenza B/Wisconsin	10^4 $TCID_{50}$/mL	-	-	-
Measles/7/2000	10^4 $TCID_{50}$/mL	-	-	-
Mumps Virus	10^4 $TCID_{50}$/mL	-	-	-
RSV A Strain Long	10^4 $TCID_{50}$/mL	-	-	-
RSV B Strain Wash	10^4 $TCID_{50}$/mL	-	-	-
Varicella Zoster Virus	10^4 $TCID_{50}$/mL	-	-	-
Bordetella pertussis	10^6 CFU/mL	-	-	-
Bordetella bronchiseptica	$5x10^7$ CFU/mL	-	-	-
Chlamydia pneumoniae	10^4 $TCID_{50}$/mL	-	-	-
Chlamydia trachomatis	$10^4$$TCID_{50}$/mL	-	-	-
Legionella pneumophila	10^6 CFU/mL	-	-	-
Mycobacterium intracellulare	10^7 CFU/mL	-	-	-
Mycobacterium tuberculosis	10^6 CFU/mL	-	-	-
Haemophilus influenza	10^6 CFU/mL	-	-	-
Pseudomonas aeruginosa	10^6 CFU/mL	-	-	-
Proteus vulgaris	10^6 CFU/mL	-	-	-
Proteus mirabilis	10^6 CFU/mL	-	-	-
Neisseria gonorrhoeae	10^6 CFU/mL	-	-	-
Neisseria meningitidis	10^6 CFU/mL	-	-	-
Neisseria mucosa	$7.4x\ 10^7$ CFU/mL	-	-	-
Klebsiella pneumoniae	10^6 CFU/mL	-	-	-
Escherichia coli	10^6 CFU/mL	-	-	-
Moraxella catarrhalis	$1.3x10^7$ CFU/mL	-	-	-
Corynebacterium diptheriae	$3x10^7$ CFU/mL	-	-	-
Lactobacillus plantarum	10^6 CFU/mL	-	-	-
Streptococcus pneumoniae	10^6 CFU/mL	-	-	-
Streptococcus pyogenes	10^6 CFU/mL	-	-	-
Streptococcus salivarius	$2x10^6$ CFU/mL	-	-	-
Staphylococcus epidermidis	10^6 CFU/mL	-	-	-
Staphylococcus aureus	10^6 CFU/mL	-	-	-
Candida albicans	10^6 CFU/mL	-	-	-

Note: Fresh cultured and titered stocks of organisms were used for the analytical specificity study, except for *C. pneumoniae* and *C.* ATCC frozen cultures and ATCC supplied titers were used for both due to technical difficulties in re-growing and re-titering these organisms.

Interference

Mucin, whole blood and a number of potentially interfering exogenous substances (medications and over the counter (OTC) products) that may be present in the nasopharynx were evaluated in the ProParaflu+ assay. HPIV-3 was spiked into HPIV negative NP pools at 2X LoD and 10X LoD. Clinically relevant amounts of the potential interfering substances were added to spiked samples. An Internal Control (IC) was also added to each sample. Nucleic acid from the samples was extracted with the Roche MagNA Pure LC instrument. The ProParaflu+ assay was performed in triplicate reactions for each sample on the Cepheid SmartCycler II. The following table shows the potential interfering substances used for this study. The substances consisted of nasal sprays (liquid and powder), ingestible pills and lozenges, injectables, and endogenous substances:

Substance Name	Active Ingredient	Concentration Tested
Mucin	Purified mucin protein	60 µg/mL
Blood (human)	N/A	2% (volume/volume)
Neo-Synephrine	Phenylephrine HCl	15% (volume/volume)
Anefrin Nasal Spray	Oxymetazoline Hydrochloride	15% (volume/volume)
Zicam Nasal gel	Luffa Operculata, Galphimia Glauca, Histaminum Hydrochloricum,	5% (volume/volume)
Saline Nasal Spray	Sodium chloride with preservatives	15% (volume/volume) of dose
Chloraseptic Throat Lozenges	Oral anesthetic/analgesic	0.63 mg/mL; active ingredients: 1.0 mg/mL benzocaine, 1.7 mg/mL menthol
Relenza	Zanamivir	3.3 mg/mL
Tobramycin	Tobramycin	4.0 µg/mL
Mupirocin	Mupirocin	6.6 mg/mL
Rebitol	Ribavirin	20 mg/mL
TamiFlu	Oseltamivir	25 mg/mL
Beconase AQ	Beclomethasone dipropionate	5% (volume/volume)

All of the exogenous or endogenous potentially interfering substances tested were found to have no effect on ProParaflu+ assay results when present in simulated respiratory samples at the 10X LoD concentration. However, some evidence of interference of HPIV detection at the LoD was observed with throat lozenges, Relenza and Rebitol, albeit these substances were tested at concentrations much higher than would be encountered *in vivo*.

Extraction Equivalency

Extraction equivalency of the bioMérieux NucliSENS easyMAG and Roche MagNA Pure LC instruments was evaluated by performing a limit of detection study. A single cultured and titered strain of HPIV-3 was spiked (along with an IC) into individual aliquots of negative NP matrix pools at concentrations of 1 log above, at, and 1 log below the previously determined LoD. Each viral strain dilution was extracted in replicates of 10 on each automated extractor and tested using the ProParaflu+ assay.

The bioMérieux NucliSens easyMAG instrument and the Roche MagNA Pure LC instrument performed equivalently with respect to limit of detection.

Carry-over/Cross-Contamination

To evaluate the degree of carry-over/cross-contamination that occurs with the use of the ProParaflu+ assay in association with nucleic acid extraction on the Roche MagNA Pure LC and the bioMérieux NucliSens easyMAG instruments and RT-PCR on the Cepheid SmartCycler II thermocycler, an internal Carry-Over study was performed. Simulated HPIV-2 high positive samples were run in series alternating with HPIV-2 high negative samples. The HPIV-2 high positive samples in this study represented the lower Cycle Threshold (Ct) range (higher sample titer range) obtained in the Pro hMPV+ clinical trials (lowest Ct = 22.8). The high-negative samples include a low amount of HPIV-2 that should be detectable no more than 5% of the time. Five (5) out of 110 (4.5%) high-negative samples tested showed potential HPIV-2 contamination when extracted using either extraction system in the study. Also some HPIV-1 and HPIV-3 contamination was also observed (n = 5 of 220) during initial testing, but was not observed when samples were retested. Potential contamination could have occurred during: creation of the sample, sample preparation for extraction, extraction, transfer of purified nucleic acid samples from the sample cartridge to microfuge tubes, or set up of the RT-PCR reaction. The possibility that these high negative samples (HPIV-2) fall in the 5% category could not be ruled out.

Disposal

Dispose of hazardous or biologically contaminated materials according to the practices of your institution.

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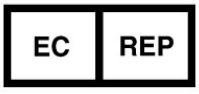
	Upper Storage Temperature Limitation		Authorized Representative
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